

## RESEARCH ARTICLE

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# Induction of early Purkinje cell dendritic differentiation by thyroid hormone requires ROR $\alpha$

Fatiha Boukhtouche<sup>1,2,3\*</sup>, Bernard Brugg<sup>1,2</sup>, Rosine Wehrlé<sup>1,2</sup>, Brigitte Bois-Joyeux<sup>4</sup>, Jean-Louis Danan<sup>4</sup>, Isabelle Dusart<sup>1,2</sup>, Jean Mariani<sup>1,2,5</sup>

## Abstract

**Background:** The active form (T<sub>3</sub>) of thyroid hormone (TH) controls critical aspects of cerebellar development, such as migration of postmitotic neurons and terminal dendritic differentiation of Purkinje cells. The effects of T<sub>3</sub> on early dendritic differentiation are poorly understood.

**Results:** In this study, we have analyzed the influence of T<sub>3</sub> on the progression of the early steps of Purkinje cell dendritic differentiation in postnatal day 0 organotypic cerebellar cultures. These steps include, successively, regression of immature neuritic processes, a stellate cell stage, and the extension of several long and mature perisomatic protrusions before the growth of the ultimate dendritic tree. We also studied the involvement of ROR $\alpha$ , a nuclear receptor controlling early Purkinje cell dendritic differentiation. We show that T<sub>3</sub> treatment leads to an accelerated progression of the early steps of dendritic differentiation in culture, together with an increased expression of ROR $\alpha$  (mRNA and protein) in both Purkinje cells and interneurons. Finally, we show that T<sub>3</sub> failed to promote early dendritic differentiation in *staggerer* ROR $\alpha$ -deficient Purkinje cells.

**Conclusions:** Our results demonstrate that T<sub>3</sub> action on the early Purkinje cell dendritic differentiation process is mediated by ROR $\alpha$ .

## Background

The thyroid hormone (TH) L-3,3',5-triiodothyronine (T<sub>3</sub>) is essential for normal central nervous system development [1], regulating processes associated with brain differentiation, such as neuronal migration, axonal and dendritic growth, synaptogenesis, and myelination [2]. In particular, TH plays an important role in cerebellar neurogenesis [3-5], a mainly postnatal developmental process. As a consequence, perinatal hypothyroidism affects the morphogenesis of cerebellar neurons (in particular the dendritic arborization of the Purkinje cells (PCs), which display a striking reduction in the growth and branching of their dendritic arborization [6]) and delays synaptic formation between PCs and granule cells [3-5,7] (for review, see [8]). Recent studies have demonstrated that THs promote this growth of the PC mature dendritic tree through activation of the nuclear thyroid hormone receptor (TR) TR $\alpha$ 1 [9,10].

Shortly after birth, cerebellar PCs display a bipolar shape reminiscent of their migratory morphology. These immature PCs then follow a process of dendritic regression, prior to extending dendrites from which the ultimate and characteristic mature dendritic tree will arise (for review, see [11]). We have recently demonstrated that the nuclear receptor Retinoic acid receptor-related orphan receptor alpha (ROR $\alpha$ , NR1D1) controls the early dendritic differentiation steps, particularly the regressive phase of this process [12]. The loss-of-function *staggerer* (sg) mutation in the *Rora* gene leads to cerebellar developmental defects in the mouse, including dramatic PC and granule cell loss [13-16]. Interestingly, cross-talk between the TH pathway and ROR $\alpha$  has been shown. In hypothyroid rats, daily thyroxine (T<sub>4</sub>) replacement accelerates the increase of ROR $\alpha$  mRNA within the developing cerebellum, most obviously at P15 [17]. In the homozygous *staggerer* mutant mouse (*Rora*<sup>sg/sg</sup>), despite both normal TR expression [14] and normal serum TH levels [18], *staggerer* neurons seem to be unresponsive to TH treatment [19].

\* Correspondence: [fatiha.boukhtouche@gmail.com](mailto:fatiha.boukhtouche@gmail.com)

<sup>1</sup>UPMC Université Paris 6, UMR 7102 NPA, F-75005, Paris, France

Despite the detailed description of cerebellar abnormalities due to hypothyroidism, most studies investigate the role of TH in the growth of the mature PC dendritic tree, which involves cross-talk and synaptogenesis with granule cells; but little is known about the effect of TH on early dendritic differentiation. In this study, we aimed at determining the role of TH in early PC dendritic differentiation, that is, during the phase of regression of the primary dendrite, and we studied the involvement of ROR $\alpha$  in this process. Using organotypic cultures, we have thus studied the progression of PC dendritic differentiation in the presence or absence of T<sub>3</sub>, and we observed an acceleration of the process of dendritic differentiation when T<sub>3</sub> was added onto postnatal day 0 (P0) cerebellar slices for both early events (regression of the primary dendrite observed after 3 days *in vitro* (DIV)) and later ones (growth of the mature dendritic tree). We further propose that the accelerated early dendritic differentiation is dependent on a T<sub>3</sub>-induced increase of ROR $\alpha$  expression.

## Results

### Determination of the optimal T<sub>3</sub> concentration to promote PC dendritic growth in organotypic culture

At birth, *in vivo*, most murine PCs are fusiform (bipolar shape, stage I; data not shown), as described for rats [20]. When cultured at P0 and kept in organotypic cultures, PCs present first an immature morphology (bipolar fusiform, stage I), then retract their primitive dendrites to become stellate or atrophic (stage II), elongate numerous long and mature dendritic perisomatic protrusions (stage III), and finally develop their ultimate dendritic trees (stage IV) [12].

In organotypic cultures, after 7 days in a serum-containing medium, PCs were mostly in stage II (stellate or atrophic stage), whereas almost no stage III PCs were observed. In order to explore the involvement of T<sub>3</sub> in dendritic differentiation (that is, before the extension of the ultimate dendritic tree in stage IV), cultures from P0 animals were prepared and kept 7 DIV under serum-free conditions, with or without addition of T<sub>3</sub> at different concentrations. Cultures were then fixed and immunolabeled with an anti-calbindin (anti-CaBP) antibody to visualize PCs.

In P0 slices cultivated without T<sub>3</sub>, most PCs (76%) displayed 'stellate or atrophic' dendrites after 7 DIV (stage II; Figure 1A,B). Adding T<sub>3</sub> at a concentration of 3 nM did not dramatically modify the repartition of PC classes (Figure 1C,D). In contrast, supply of 30 nM of T<sub>3</sub> led to a significant acceleration of the dendritic differentiation since we observed that only 27% of PCs were in stage II, whereas 39% displayed long dendritic perisomatic protrusions and were thus classified as stage III PCs, and 34% displayed an identified mature dendritic tree (stage IV;

Figure 1E,F). Increased concentration of T<sub>3</sub> (100 nM) also led to an acceleration of the dendritic differentiation (compared to 0 nM and 3 nM of T<sub>3</sub>), although this treatment was not as efficient as 30 nM T<sub>3</sub> since the proportion of stage IV PCs was lower (Figure 1G,H). Thus, these results show that, in organotypic culture, as already demonstrated for dissociated cell culture [10,11], the addition of T<sub>3</sub> to the culture medium promotes PC dendritic development in a dose-dependent manner. In the following experiments, we have used the 30 nM concentration to assess the effects of T<sub>3</sub> since this concentration is the most efficient in our culture conditions.

### T<sub>3</sub> leads to an increased amount of ROR $\alpha$ protein in PCs and interneurons

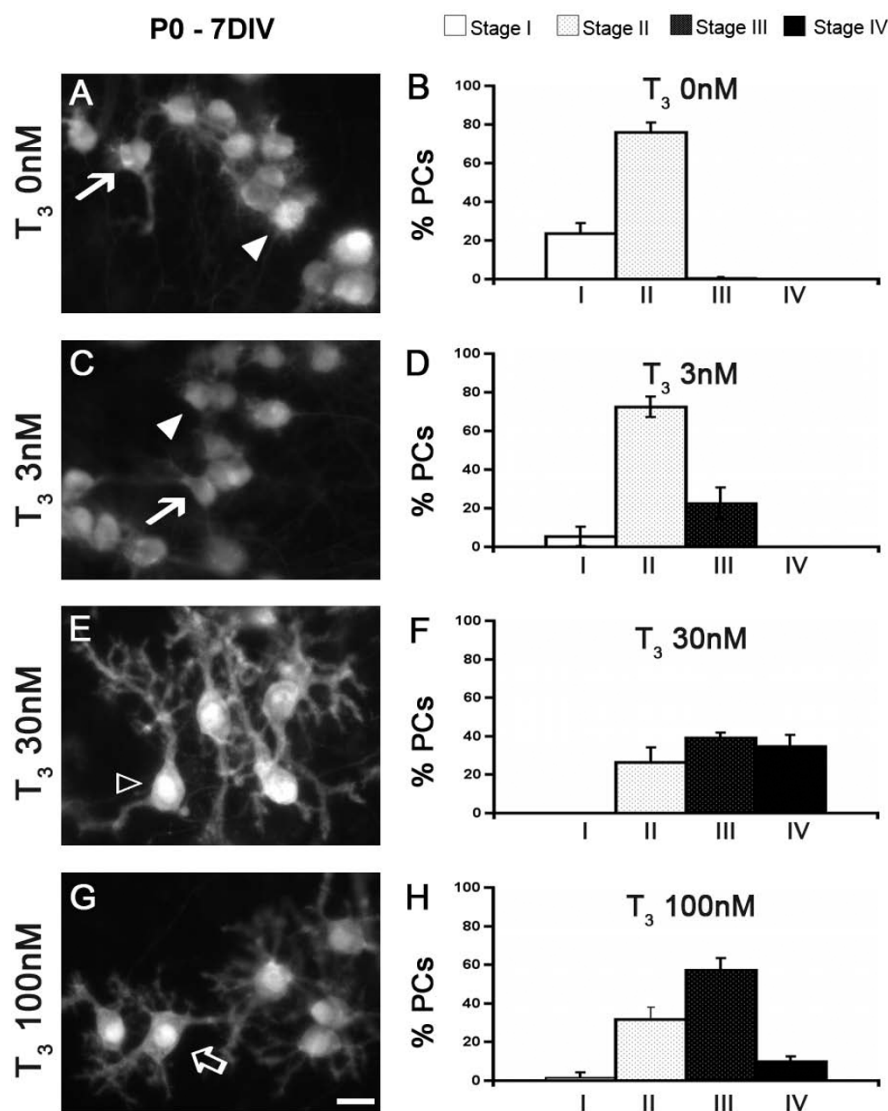
To determine whether the T<sub>3</sub>-induced acceleration of dendritic differentiation involves ROR $\alpha$ , we first assessed ROR $\alpha$  expression in cerebellar slices in response to T<sub>3</sub> treatment. By combination of promoter usage and alternative splicing, the *Rora* gene encodes two isoforms in the mouse (ROR $\alpha$ 1 and ROR $\alpha$ 4), which differ only in their amino-terminal modulator region [21-23].

Western blots of P0 7 DIV cerebellar slices were performed with antibodies directed against the carboxyl terminus of ROR $\alpha$ , which can detect both ROR $\alpha$ 1 and ROR $\alpha$ 4 isoforms. We detected an increase of 6.8-fold in the amount of ROR $\alpha$ 1 protein in slices treated with 30 nM of T<sub>3</sub> (Figure 2A).

In the cerebellum, ROR $\alpha$  is known to be expressed only in PCs and interneurons [24]. In order to determine in which cell type the upregulation of ROR $\alpha$  expression occurs, we used immunofluorescence to detect and locate the ROR $\alpha$  protein in organotypic cultures. Since only PCs in the cerebellum express CaBP, we used CaBP as a PC-specific marker, and we used parvalbumin as a marker of interneurons. Both mature interneurons and PCs express parvalbumin: interneurons were unambiguously identified as parvalbumin-positive and CaBP-negative cells.

To assess whether T<sub>3</sub> led to increased expression ROR $\alpha$ 1 in PCs, we quantified the fluorescence density of ROR $\alpha$  labeling within the nucleus of PCs. We observed a significant increase in the fluorescence density in T<sub>3</sub>-treated slices compared to control T<sub>3</sub>-untreated slices (Figure 2B). Interestingly, following T<sub>3</sub> treatment, ROR $\alpha$  labeling was observed in PCs and also in some CaBP-negative cells. Some CaBP-negative cells that express ROR $\alpha$  also expressed parvalbumin, and were thereby identified as interneurons (Figure 2B). Therefore, T<sub>3</sub> treatment led to increased expression of ROR $\alpha$  in both PCs and parvalbumin-positive interneurons.

To determine whether the increase in ROR $\alpha$  protein levels in T<sub>3</sub>-treated cerebellar slices is the consequence of



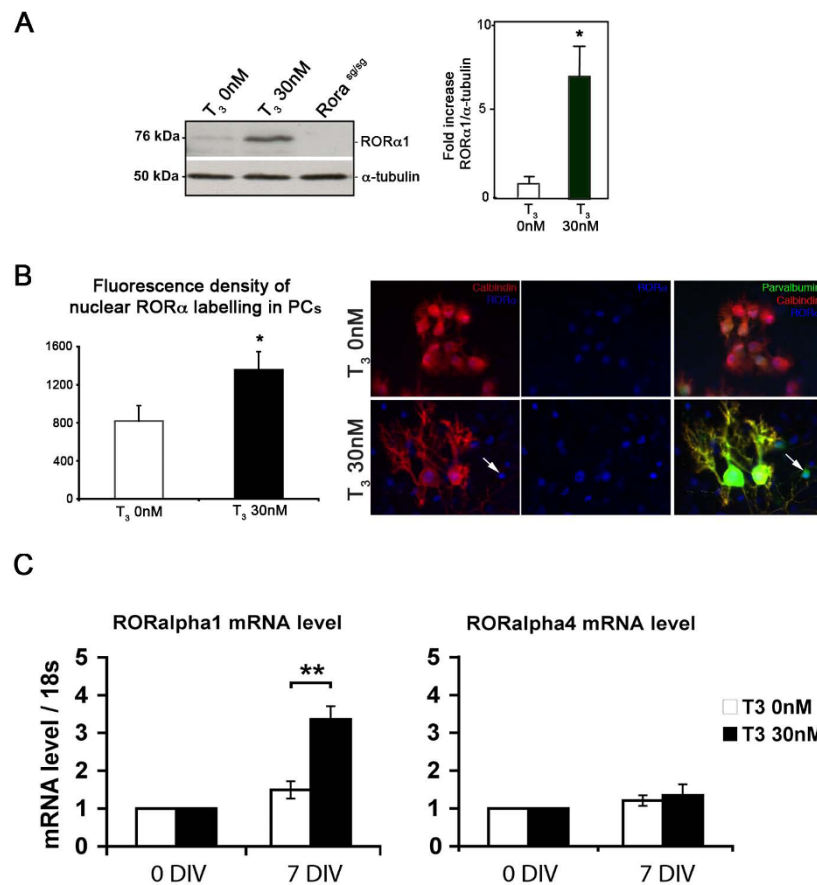
**Figure 1 Dose-dependent effect of T<sub>3</sub> on PC dendritic differentiation in organotypic cultures. (A-H).** Organotypic cultures of P0 cerebella were kept 7 DIV in the absence of T<sub>3</sub> (A,B), or in the presence of 3 nM T<sub>3</sub> (C,D), 30 nM T<sub>3</sub> (E,F) or 100 nM T<sub>3</sub> (G,H). (A,C,E,G) PCs were revealed by CaBP immunolabeling. (B,D,F,H) Quantitative distribution of PCs between stages I to IV. Fusiform PCs with a bipolar shape are defined as stage I (arrow in (A,C)), PCs with regressive atrophic dendrites all around the soma are defined as stage II (white arrowhead in (A,C)), PCs with one or more perisomatic protrusions are defined as stage III (empty arrow in (G)) and PCs with an identified dendritic tree are classified as stage IV (empty arrowhead in (E)). Scale bar = 20  $\mu$ m. Error bars indicate mean  $\pm$  standard deviation.

increased expression of the *Rora* gene, and not stabilization of the protein, we analyzed by real time RT-PCR the mRNA level of the different *Rora* isoforms in the T<sub>3</sub>-treated slices compared to untreated slices after 7 DIV (Figure 2C). A 3.3-fold increase was observed for *Rora1* after 7 DIV. The *Rora4* mRNA level was similar to untreated slices after 7 DIV. These results show that T<sub>3</sub> leads to increased expression of the *Rora1* isoform after 7 DIV.

These results show that T<sub>3</sub> induced increased ROR $\alpha$ 1 protein levels in PCs in parallel with their dendritic differentiation after 7 DIV.

#### T<sub>3</sub> accelerates the first steps of early PC dendritic differentiation and increases *Rora* gene expression at P0

As we previously demonstrated that ROR $\alpha$  is involved in early dendritic differentiation [12], we examined whether T<sub>3</sub> promotes this early change. We thus assessed the effect of T<sub>3</sub> treatment on cerebellar slices after 3 DIV, a time when PCs cultured without T<sub>3</sub> display mainly bipolar fusiform dendritic morphology (stage I; 97%) whereas very few are in a stellate or atrophic morphology (stage II; 3%; Figure 3A). In the presence of 30 nM of T<sub>3</sub> after 3 DIV, all PCs were still



**Figure 2 T<sub>3</sub> treatment increases the amount of RORα protein and RNA in organotypic cultures.** P0 cerebellar slices kept for 7 DIV were cultured in the absence or the presence of 30 nM T<sub>3</sub>. **(A)** Immunoblot analysis and quantification of RORα levels in extracts of untreated or T<sub>3</sub>-treated cerebellar slices (\**P* < 0.05). **(B)** Left panel: fluorescence density of RORα immunolabeling was measured within each PC nucleus with MetaMorph software. Average values from multiple cells ± SEM are shown (\**P* < 0.05). Right panel: organotypic cultures after 10 DIV without T<sub>3</sub> (upper row) or with T<sub>3</sub> (30 nM) treatment (lower row). RORα-expressing cells were revealed by RORα immunolabeling (blue), PCs were revealed by CaBP immunolabeling (red) and both PCs and interneurons were revealed by parvalbumin immunolabeling (green). Note the presence of RORα-expressing interneurons (arrow) in the T<sub>3</sub> only treatment. **(C)** P0 organotypic cultures were cultured without T<sub>3</sub> (white bars) or with 30 nM T<sub>3</sub> (black bars) for 7 days. Levels of mRNA were determined by real time RT-PCR and standardized to 18 s rRNA. The data are given relative to the mRNA level in untreated slices at the initial time of the culture (0 DIV). They were obtained from three independent cerebellar slices extracts (\**P* < 0.05; \*\**P* < 0.005). Error bars in (C) indicate mean ± standard deviation.

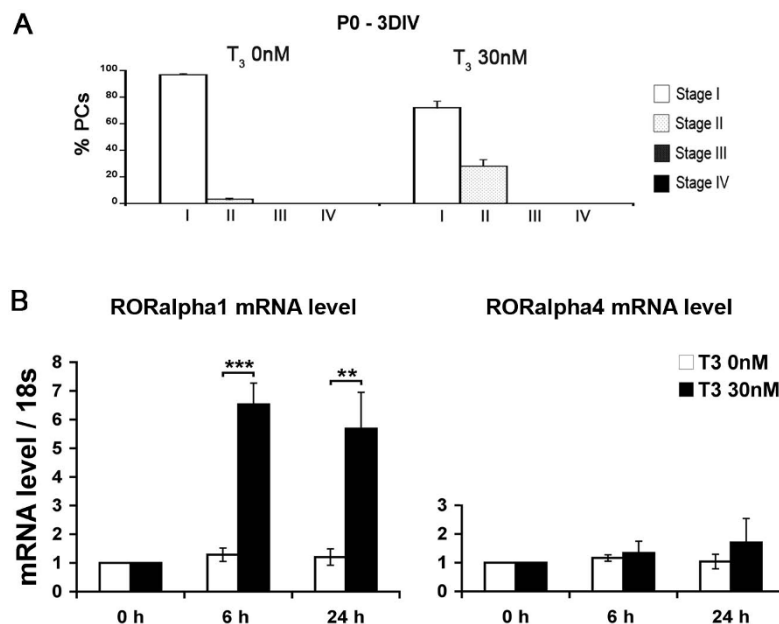
in stage I or II, but we observed an increased number of PCs in stage II (28%; Figure 3A) compared to the control without T<sub>3</sub> (3%; Figure 3A). From those experiments, we can conclude that T<sub>3</sub> promotes the first dendritic differentiation steps of PCs from stage I to stage II in organotypic cultures.

To determine whether T<sub>3</sub> increases *Rora* expression in early stages of PC development, we analyzed the mRNA levels of the *Rora* isoforms in response to T<sub>3</sub> treatment during the first day of culture (Figure 3B). We observed a specific increase in the *Rora1* mRNA level after 6 h and 24 h of T<sub>3</sub> treatment (6.5- and 5.7-fold increase, respectively). The *Rora4* mRNA level was slightly and transiently increased in slices after 24 h of T<sub>3</sub> treatment (1.7-fold increase). These results show that T<sub>3</sub> leads to

increased expression of the *Rora1* isoform in fusiform PCs at P0 and, to a lesser extent, of the *Rora4* isoform. Interestingly, our results also revealed that mRNA levels of both *Rora1* and *Rora4* isoforms were stable in cultures made at P0 and kept for 6 h, 24 h and 7 DIV in culture without T<sub>3</sub> (compare Figures 2C and 3B).

#### T<sub>3</sub>-induced early dendritic differentiation involves RORα

The experiments described above show that T<sub>3</sub> promotes dendritic differentiation (Figures 1 and 3) and leads to increased expression of RORα1 (Figures 2 and 3). We have recently shown that RORα is a crucial factor controlling the early steps of PC dendritic differentiation, and *staggerer* RORα-deficient PCs do not progress beyond early bipolar migratory morphology



**Figure 3**  $T_3$  promotes the early dendritic differentiation of PCs and leads to increased mRNA levels of *Rora1* and *Rora4* at P0. **(A)** Quantitative distribution of PCs between stages I and IV. Cultures of P0 cerebella were kept 3 DIV in the absence or the presence of 30 nM  $T_3$ . PCs are classified following the same criteria as in Figure 1. **(B)** P0 organotypic cultures were cultured without  $T_3$  (white bars) or with 30 nM  $T_3$  (black bars). Levels of mRNA were determined by real time RT-PCR and standardized to 18 s rRNA after 0 h, 6 h or 24 h of  $T_3$  treatment. The data are given relative to the mRNA level in untreated slices at the initial time of the culture (0 h). They were obtained from three independent cerebellar slice extracts (\*\* $P < 0.005$ ; \*\*\*  $P < 0.0005$ ). Error bars indicate mean  $\pm$  standard deviation.

[12]. To determine whether  $ROR\alpha$  is actually involved in the  $T_3$ -induced PC dendritic differentiation, we followed the progression of the dendritic differentiation of PCs from *staggerer* ( $Rora^{sg/sg}$ ) and corresponding control  $Rora^{+/+}$  cerebellar slices treated or not with  $T_3$ .

As previously observed in serum-containing cultures [12], PCs from  $Rora^{sg/sg}$  cultured in serum-free medium display the embryonic bipolar shape (stage I) after 7 DIV (Figure 4B), whereas most PCs in control  $Rora^{+/+}$  cultures display 'regressive-atrophic' dendrites (stage II; Figure 4A). In the presence of 30 nM  $T_3$ , stage II, III and IV PCs were found (Figure 4C) with the same proportions as described in Figure 1. In contrast, PCs from  $Rora^{sg/sg}$  animals still display embryonic bipolar shape (Figure 4D) with long processes characteristic of stage I PCs [12], indicating that they were not responsive to  $T_3$  treatment and remained in the very early stage of dendritic differentiation.

The absence of a functional  $ROR\alpha$  protein thus prevents  $T_3$ -induced accelerated dendritic differentiation of immature bipolar P0 PCs. This experiment shows that  $ROR\alpha$  is required in the  $T_3$ -induced dendritic differentiation-promoting process.

#### $T_3$ up-regulates the activity of the *Rora* promoter

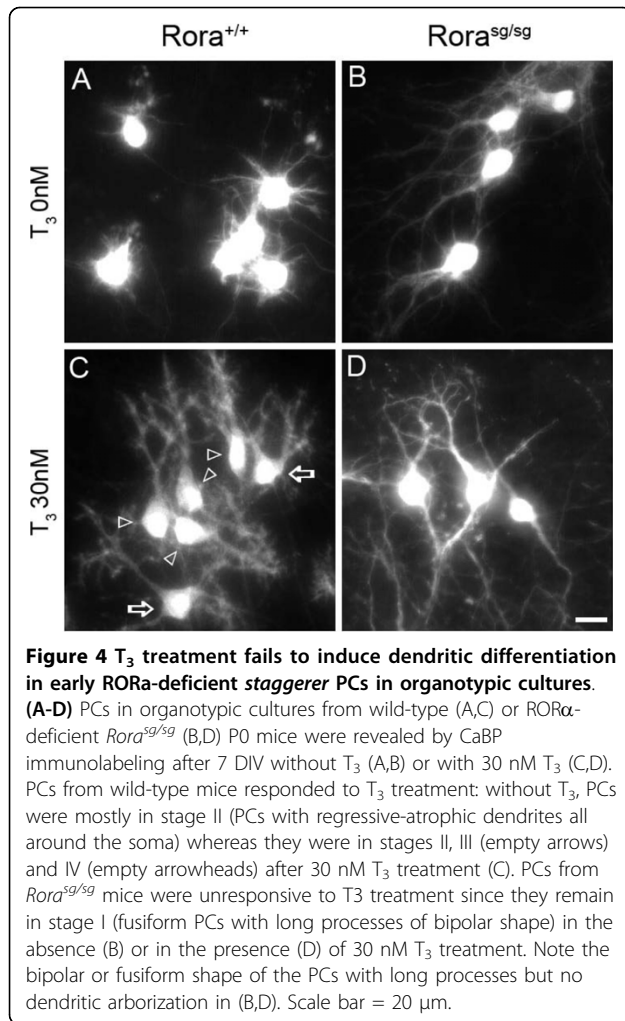
To gain further insight into the mechanism by which  $T_3$  up-regulates *Rora* gene expression, we tested the effect

of  $T_3$  on the transcriptional activity of the p(-487)*Rora*-Luc construct in HepG2 cells, which shows 82.9% sequence homology with the murine sequence and has been previously used as a model to analyze the transcriptional regulation of the *Rora* gene [25] (Additional file 1).  $T_3$  treatment resulted in a 3.6-fold increase in the activity of the p(-487)*Rora*-Luc construct compared to its basal activity in the absence of  $T_3$  (Figure 5). Plasmid pDR4-TK-Luc, which contains a thyroid receptor response element (DR4), was used as a control for the effect of  $T_3$  on transcriptional activity. As expected, the luciferase activity of pDR4-TK-Luc was strongly increased (12.1-fold) by  $T_3$  treatment, indicating that  $T_3$  is active under our experimental conditions.

Taken together, the results of these experiments indicate that the effect of  $T_3$  on *Rora* expression is, at least in part, transcriptional and that the -487 to -45 *Rora* promoter region is involved in this regulation.

#### Discussion

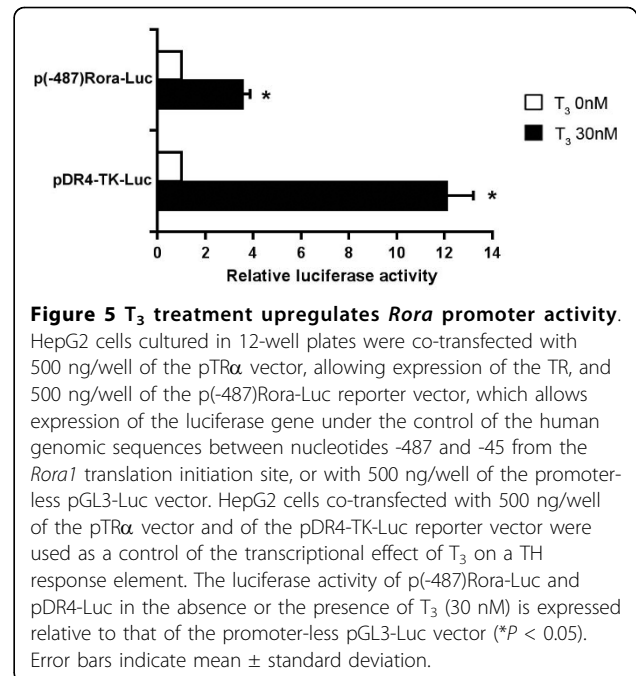
Our results show for the first time that  $T_3$  promotes the early steps of PC dendritic differentiation, during the phase of neurite regression that precedes the formation of the ultimate dendritic tree. Addition of  $T_3$  to the serum-free medium of P0 cerebellar slices resulted in an acceleration of the early steps of dendritic differentiation. This accelerated progression of dendritic



differentiation was accompanied by increased expression of the gene encoding the nuclear receptor ROR $\alpha$ , observed at both mRNA and protein levels. We further show that the ROR $\alpha$  protein is required for the  $T_3$ -induced early dendritic differentiation, as  $T_3$  treatment did not promote dendritic differentiation in *Rora*<sup>sg/sg</sup> PCs. This result is in accordance with previous studies that suggest an unresponsiveness of *Rora*<sup>sg/sg</sup> mutants to TH [19].

#### $T_3$ promotes early PC dendritic differentiation

The role of TH in mammalian brain is well documented, particularly during cerebellar development (for review, see [1,26]). Congenital hypothyroidism in humans leads to a syndrome termed cretinism [27], the apparent symptoms of which include ataxia and poor motor skills, indicating cerebellar defects. In PCs, TH is known to strongly promote differentiation of the elaborate dendritic tree and synapse formation. In contrast, little is known about its role in the events preceding the



development of the ultimate dendritic tree, in particular the steps of neuritic regression and early extension of perisomatic protrusions, occurring *in vivo* in the rodent between P0 and P7.

To better understand the effect of TH action in the developing brain, the temporal patterns of initiation and cessation of hormone action need to be determined. Most *in vitro* or *in vivo* experiments explore the effects of hypo- or hyperthyroidism in the cerebellum from P15, or its equivalent age in culture. At this age, the characteristic shape of the dendritic arborization is already achieved, and extrinsic factors such as electrical activity [28] from granule cells, trophic factors [29-32] and TH modulate the growth of the dendritic arborization [4]. Studies have shown a role for TH in the persistence of the external granular layer and the migration of granule cells into the internal granular layer [4,5,33], in the proliferation and differentiation of interneurons [34], as well as a direct role of TH on PCs through TR $\alpha$ 1 receptor activation [10]. However, the effects of TH on PC dendritic differentiation during early steps that do not require cell-cell interaction have not been shown.

Using P0 slices after 3 DIV, we could specifically assess the role of TH in early development, and our results show that  $T_3$  also plays a key role in the early dendritic differentiation of PCs in organotypic cultures, that is, before the formation of the elaborated dendritic tree. These data extend the well-known role of  $T_3$  in the later stages of PC dendritic differentiation [6,8-10] and identify a third molecule besides ROR $\alpha$  and SCLIP [12,35] involved in the first steps of PC dendritic

differentiation. Interestingly, T<sub>3</sub> is the first extrinsic factor described to play a role in these processes.

We also show that T<sub>3</sub> promotes the expression of both ROR $\alpha$  and parvalbumin in interneurons, which corroborates recent results from Manzano *et al.* [34], who have shown that TH acts on the proliferation and differentiation of interneuron precursors in the cerebellum [34]. Further studies will be required to determine whether the TH action on interneurons is mediated by ROR $\alpha$ .

#### Cross-talk between ROR $\alpha$ and the TH pathway

Interestingly, T<sub>3</sub> addition led to increased expression of ROR $\alpha$  in the cerebellar PCs and interneurons. This result is in accordance with previous studies that showed decreased expression of ROR $\alpha$  in the cerebellum of hypothyroid rats [36], whereas T<sub>4</sub> replacement led to increased expression [17].

Our results indicate that ROR $\alpha$ 1 expression is required for the T<sub>3</sub>-induced effect on early dendritic differentiation. Further, we show that the activity of the *Rora* promoter was enhanced by T<sub>3</sub> treatment in culture, suggesting that TH acts on the process of early dendritic differentiation through increased expression of the *Rora* gene. TH binds to the nuclear TH receptor (TR), a ligand-regulated transcription factor, which then binds to a target DNA sequence known as a TH response element (TRE) within the promoter region of target genes. Further studies are needed to determine whether ROR $\alpha$  is a target gene of TR, and whether the transcriptional effect observed in our study is under direct control. An additional level of interaction between ROR $\alpha$  and TR has been demonstrated by Koibuchi and collaborators [37,38], who showed that ROR $\alpha$ 1 increases TR-induced transactivation on several TREs. This could account for the ROR $\alpha$  requirement in the T<sub>3</sub>-mediated promotion of PC dendritic differentiation observed in this study. TR binds as a monomer, homodimer, or heterodimer (particularly with retinoid  $\times$  receptors) to the TRE, which is composed of two half-site core motifs (AGGTCA) with specific nucleotide spacing and orientation. ROR $\alpha$  binds as a monomer to a consensus motif composed of a 6-bp AT-rich sequence 5' to a half-site core motif, AGGTCA (ROR-response element, RORE), to activate transcription [23]. Both TR and ROR $\alpha$  are thus transcription factors that share the common core motif within their response elements. ROR $\alpha$ 1 is able to bind as a monomer to one of two core motifs (AGGTCA) of a TRE that is preceded by an AT-rich sequence [23,37]. This suggests that a subset of natural TREs containing appropriate AT-rich sequences could serve as dual-response elements for TR and ROR $\alpha$ . Because of the high homology between the human and murine ROR $\alpha$ 1 coding and promoter sequences, it is possible that ROR $\alpha$  mediates some TH actions in

human. Beside its roles in the developing cerebellum, ROR $\alpha$  has also been shown to play critical roles in many different tissues and systems, including immunity, cancer, cellular metabolism, circadian rhythm, development and ageing (for review, see [39]). Understanding the roles of ROR $\alpha$  could therefore provide further information about the pleiotropic effects of late prenatal or early postnatal hypo- and hyperthyroidism in humans.

#### Intrinsic effect of ROR $\alpha$ , and potential coordination with TH in the PC dendritic differentiation process

ROR $\alpha$  has been shown to be crucial for the progression of early differentiation of PCs in a cell-autonomous manner [12]. In cerebellar slices, T<sub>3</sub> is likely to act on ROR $\alpha$  expression within PCs. Our results extend previous studies of Heuer and Mason [10], which clearly demonstrated that PCs are a direct target of TH action through activation of TR $\alpha$ 1: TH promotes the late stages of the elaboration of PC dendritic arborization, which is also dependent upon granule cell differentiation and synaptogenesis. Interestingly, ROR $\alpha$  has been shown to control the expression of *Sonic hedgehog* (*Shh*) in PCs, which in turn promotes the proliferation of granule cells precursors in the external granular layer [40]. Thus, a coordinate mechanism involving ROR $\alpha$  and TH in cerebellar development can be proposed in which both T<sub>3</sub> and ROR $\alpha$  act on PC dendritic differentiation directly as well as indirectly via the promoting effect on granule cell development. However, the later and direct effects of T<sub>3</sub> on early PC differentiation are unlikely to be mediated by ROR $\alpha$  since we have shown that ROR $\alpha$  does not influence this later step of differentiation [12].

In its homozygous state, the murine *staggerer* mutation of the *Rora* gene leads to cerebellar atrophy due to the degeneration of most PCs [13,15,41-43]. Several histological studies of the *Rora*<sup>sg/sg</sup> cerebellum show that the remaining PCs are immature and display atrophic dendrites, devoid of spines [44-46]. These abnormalities of dendritic differentiation observed in homozygous *staggerer* mice are similar to, but worse than, those observed in hypothyroid rats. This implies that ROR $\alpha$  acts on additional processes in cerebellar development, apart from those induced by THs. This hypothesis is strengthened by the recently demonstrated neuroprotective role of ROR $\alpha$  at least partly through its control of oxidative stress mechanisms [16,47].

In conclusion, our results show that ROR $\alpha$  plays a critical role in the early T<sub>3</sub>-induced dendritic differentiation of PCs.

#### Materials and methods

##### Animals

Animal housing and all procedures were carried out in accordance with the guidelines of the French Ministry

of Agriculture and the European Community. Swiss mice were obtained from Janvier (Le Genest-St-Isle, France). The *staggerer Rora<sup>sg/sg</sup>* mutant mice were maintained on a C57BL/6J genetic background in our colony. *Rora<sup>sg/sg</sup>* and their *Rora<sup>+/+</sup>* littermates were obtained by intercrossing fertile heterozygous *Rora<sup>+/sg</sup>* animals, and were genotyped by PCR, as previously described [12].

#### Organotypic slice cultures

Swiss mice at P0 were used. Organotypic cultures of cerebellum were prepared as described previously [48]. Briefly, after decapitation, brains were dissected out into cold Gey's balanced salt solution (Sigma, Lyon, France) supplemented with 5 mg/ml glucose, and the meninges were removed. Parasagittal cerebellar slices (350  $\mu$ m thick) were cut on a McIlwain tissue chopper (Stoetling Europe, Dublin, Ireland) and transferred onto 30 mm Millipore membrane culture inserts with a 0.4  $\mu$ m pore size (Millicell CM, Millipore, Molsheim, France). Slices were maintained in culture in six-well plates containing 1 ml per well of medium containing basal medium with Earle's salts (BME), supplemented with Sigma I-1884 supplement (1:100 dilution, resulting in final concentrations of 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, and 5 ng/ml sodium selenite), 0.5  $\mu$ g/ml BSA (Sigma), 4 mM L-glutamine (Invitrogen, GIBCO, Cergy Pontoise, France), 5 mg/ml glucose, with or without T<sub>3</sub> at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The medium was replaced every 2 days (after 2, 4 and 6 days in culture).

Mice obtained from *Rora<sup>+/sg</sup>* intercrosses were also used in this study. In these litters, *Rora<sup>+/+</sup>*, *Rora<sup>+/sg</sup>* and *Rora<sup>sg/sg</sup>* mice could be generated. For each animal, slices of each cerebellum were divided between two Millicells: half of the cerebellar slices served as controls and no T<sub>3</sub> was added and the other half were treated with T<sub>3</sub> (30 nM) in order to compare control (0 nM T<sub>3</sub>) versus T<sub>3</sub>-treated slices (30 nM T<sub>3</sub>) from the same animals. The genotype was determined *a posteriori* by PCR on tail biopsy, in blind studies.

#### Antibodies and staining procedures

Immunostaining of CaBP, parvalbumin or ROR $\alpha$  was performed as described previously [12]. Briefly, cerebellar slices were fixed in 4% paraformaldehyde, and then incubated for 1 h in phosphate-buffered saline containing 0.25% Triton X-100, 0.2% gelatin, 0.1% sodium azide (PBSGTA) and 0.1 M lysine. Rabbit polyclonal or mouse anti-CaBP antibody (1:5,000 dilution; Swant, Switzerland) to visualize PCs, or rabbit polyclonal anti-parvalbumin (1:5,000 dilution; Swant) to visualize both PCs and interneurons, and goat polyclonal anti-ROR $\alpha$ 1 (sc-6062; 1:4,000 dilution; Santa-Cruz, Tebu-Bio SA, Le Perray en Yvelines, France) in PBSGTA were applied

overnight. At this dilution, the intensity of ROR $\alpha$  labeling was correlated to the ROR $\alpha$  expression level [12]. Specific labeling was detected with Cy3-conjugated donkey anti-rabbit antibody (1:500 dilution; Jackson ImmunoResearch, Immunotech, Marseille, France) and FITC-conjugated donkey anti-goat antibody (1:2,000 dilution; Jackson ImmunoResearch). The slices were analyzed with an inverted microscope (Nikon Eclipse TE 300). Immunofluorescence images were captured at 400 $\times$  magnification using a Qimaging Retiga 1300 camera, and analyzed using Image-Pro Plus 4.1 software (Media Cybernetics, Bethesda, MD, USA). For ROR $\alpha$  fluorescence intensity measurements, fluorescence density was measured in the nucleus of PCs (visualized by CaBP immunolabeling) using MetaMorph software.

#### Classification of PC dendritic differentiation stages

Classification of PCs was assessed after CaBP immunostaining, as previously described [12]. Briefly, fusiform PCs with a bipolar shape, reminiscent of embryonic migratory PCs, are defined as stage I and correspond to both 'simple' and 'complex' fusiform stages, observed from embryonic day 16 to P4 *in vivo* [20]. Stage II comprises PCs with short processes all around the soma. This 'stellate' stage includes both 'regressive-atrophic dendrites' and 'stellate cell' stages described previously, from P2 to P6 *in vivo*. PCs with more than one long and mature dendritic protrusion are defined as stage III. They correspond to PCs around P5 to P10 *in vivo*. Finally, PCs with one well identified dendritic tree (defined as primary dendrites giving rise to additional side branches) are classified as stage IV. Images were taken from all slices, corresponding to at least 200 PCs in each experiment. Quantification was performed on three independent experiments.

#### Western-blot analysis

Cultured slices were lysed in solubilization buffer (500 mM NaCl, 1 mM MgCl<sub>2</sub>, 2 mM EGTA, 50 mM Bicine, pH 9.0, 50 mM NaF, 5  $\mu$ M ZnCl<sub>2</sub>, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM dithiothreitol, 5 nM okadaic acid, 2.5  $\mu$ g/ml aprotinin, 3.6  $\mu$ M pepstatin, 0.5  $\mu$ M phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 5.3  $\mu$ M leupeptin) and dounced at 4°C. Insoluble materials were removed by centrifugation (13,000 g for 20 minutes at 4°C), supernatants were isolated and the samples were stored at -80°C. Proteins were dosed with the DC protein assay kit (Bio-Rad, Hercules, CA, USA). As previously described [49], cell-extracts containing equivalent amounts of protein were boiled for 5 minutes in sample loading buffer. After a 10% SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (ICN Biochemicals, Costa Mesa, CA USA). Non-specific sites were blocked with 5% skimmed dried



milk for 2 h. Blots were then incubated overnight at 4°C with primary antibodies against ROR $\alpha$  (1:2,000; Santa Cruz) and  $\alpha$ -tubulin (1:10,000; Sigma) in 5% skimmed dried milk. They were then incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skimmed dried milk for 1 h. The revelation was processed with enhanced chemoluminescence substrate (Amersham, Saclay, France). Quantification was performed using Densylab software (Microvision Instruments, Evry, France).

### Real-time RT-PCR

Total RNA from cerebellar slices from three animals was prepared according to the manufacturer's instructions using the RNeasy kit (Qiagen, Courtaboeuf, France) and cDNAs were synthesized from 1  $\mu$ g of RNA (Promega, Charbonnières-les-Bains, France) and avian myeloblastosis virus (AMV) reverse transcriptase, as per the manufacturer's instructions.

RT-PCR was performed using the ABsolute™ QPCR SYBR® Green Mixes Kit (ABgene, Courtaboeuf, France), as per the manufacturer's instructions. Reactions were performed in 25  $\mu$ l of total volume containing ABsolute™ QPCR SYBR® Green Mix with 8 ng of the first-strand cDNA and 300 nM of primers. The following primers were used: *Rora1* sense, 5'-AGGCA-GAGCTATGCGAGC-3', and antisense, 5'-TCAAACAGTTCTTCTGACGAGG-3'; *Rora4* sense, 5'-GTCACATGGAGCC TCTTATGG-3', and antisense, 5'-TCAAACAGTT CTTCTGACGAGG-3'; 18 s sense, 5'-GGGAGCCTGAGAAACGGC-3', and antisense, 5' GGGTCGGA GTGGGTAATTT-3'. Amplification was performed on an iCycler (Bio-Rad) according to the manufacturer's instructions and cycle parameters were: 50°C (2 minutes) and 95°C (10 minutes), followed by 40 cycles of 95°C (15 s), 60°C (30 s) and 72°C (30 s). For expression quantification, a comparative  $C_T$  method was used [50,51]. The  $\Delta C_T$  value was obtained by subtracting the  $C_T$  value of the 18 S (reference) from the  $C_T$  value of the gene of interest, where in each case the mean value of three reactions was used. For each gene, the fold change was calculated according to the formula  $2^{(-\Delta C_T)}$ , where  $\Delta C_T$  was the difference between the  $\Delta C_T$  of T3-treated cultures and the  $\Delta C_T$  of untreated cultures as a calibrator value. To distinguish specific amplicons from non-specific amplifications, a dissociation curve was generated for each transcript. Quantification was performed on three independent experiments.

### Vectors, transient transfection and luciferase assay

The plasmid p(-487)Rora-Luc contains the luciferase reporter gene placed under the control of the promoter region of the human *Rora* gene, from -487 to -45

relative to the *Rora* translation initiation site [25]. The vector pTR $\alpha$ , containing mouse *TR $\alpha$ 1* cDNA, cloned in plasmid pSG5 and plasmid pDR4-TK-Luc, which contains a TRE in front of the promoter of the thymidine kinase gene of the herpes simplex virus controlling expression of the luciferase gene where kind gifts of Dr F Flamand (Ecole Normale Supérieure, Lyon, France).

The promoter-less pGL3-basic luciferase reporter vector (pGL3-Luc) was from Promega. Transient transfection experiments were done with HepG2 human hepatoma cells using the calcium phosphate method. Twenty-four hours after the transfection, 30 nM of T<sub>3</sub> were added to the medium and the luciferase activity was assayed 24 h later, as described [25]. Activities corresponding to cells cultured with 30 nM of T<sub>3</sub> were expressed relative to those of control cells cultured without T<sub>3</sub>.

### Statistical analysis

Independent experiments were performed with 10 to 12 cerebellar slices per sample and repeated three times using matched controls. For PC stage quantification, at least 200 PCs were analyzed in each sample. For the ROR $\alpha$  RNA level quantification by real-time PCR, all slices of three animals were used in each experiment. Results are expressed in Figures as mean  $\pm$  standard deviation. The statistical significance of differences between control and T<sub>3</sub>-treated slices was assessed by a Student's *t*-test using Statview software (SAS Institute Inc., Berkeley, CA, USA).

### Additional material

**Additional file 1: Sequence comparison of the human and mouse *RORA1* promoter region.** Human and mouse sequences of the immediately upstream region of the translation initiation codon (+1) of the *Rora* gene and including the -487 to -45 *Rora* promoter region (boxed area) were aligned using ClustalW software. The sequence downstream of the initiation codon corresponds to the beginning of exon 1. The nucleotide sharing identity across both species are indicated by asterisks and gaps are indicated with hyphens. The -487 to -45 human sequence shows 82.9% identity across species. Both human and murine sequences were obtained from the GenBank database: *Homo sapiens* chromosome 15 genomic contig [NT\_010194.17], 32312505 to 32311809 bp; *Mus musculus* chromosome 9 genomic contig, strain C57BL/6J [NT\_039474.7] [Mm9\_39514\_37], 14921696 to 14922363 bp).

### Abbreviations

bp: base pair; CaBP: calbindin; DIV: days *in vitro*; P: postnatal day; PC: Purkinje cell; ROR $\alpha$ : Retinoic acid receptor-related orphan receptor alpha; sg: staggerer; T<sub>3</sub>: L-3,3',5-triiodothyronine; TH: thyroid hormone; TR: thyroid hormone receptor; TRE: TH response element.

### Acknowledgements

This research was supported by grants from Fondation pour la Recherche Médicale (FB), Fondation Lejeune (FB) and ANR-07-NEURO-043-01 (ID). We thank Rachel Sherrard for helping us with the manuscript.

## Author details

<sup>1</sup>UPMC Université Paris 6, UMR 7102 NPA, F-75005, Paris, France. <sup>2</sup>CNRS, UMR 7102 NPA, F-75005, Paris, France. <sup>3</sup>Biozentrum, Department of Cell Biology, University of Basel, CH-4056 Basel, Switzerland. <sup>4</sup>Faculté de Médecine Paris Descartes, site Necker, FRE CNRS 3210, F-75015, Paris, France. <sup>5</sup>Hôpital Charles Foix, UEF, F-94205, Ivry-sur-Seine, France.

## Authors' contributions

FB conceived of the study, designed and conducted experiments, and wrote the manuscript. RW and BBJ contributed to experiments; BB contributed to experiments and helped edit the manuscript. JLD, ID and JM supervised the study, and participated in its design and coordination, and helped edit the manuscript.

## Competing interests

The authors declare that they have no competing interests.

Received: 25 April 2010 Accepted: 27 July 2010 Published: 27 July 2010

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doi:10.1186/1749-8104-5-18

**Cite this article as:** Boukhtouche *et al.*: Induction of early Purkinje cell dendritic differentiation by thyroid hormone requires ROR $\alpha$ . *Neural Development* 2010 **5**:18.

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